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Simple Green Purification of Spilanthol from Natural Deep Eutectic Solvent and Ethanolic *Acmella oleracea* (L.) R.K. Jansen Extracts Using Solid-Phase Extraction

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Abstract: Spilanthol is a major *N*-alkylamide constituent of *Acmella oleracea* (L.) R.K. Jansen with diverse pharmacological properties. We recently showed the applicability of NADES (natural deep eutectic solvents) for the green extraction of spilanthol. However, the purification of targets from NADES poses a challenging step due to their non-volatility. A simple green method to retrieve spilanthol with minimal instrumental effort was devised, fractioning NADES (choline chloride/methylurea, choline chloride/1,2-propanediol, choline chloride/citric acid) and dry ethanolic extracts by SPE on C18 material, eluting merely with ethanolic solutions. The relative distribution of spilanthol and organic adulteration in SPE fractions were detected by HPLC-DAD, followed by scale-up, quantification and purity determination in an NMR-based approach. Isocratic elution with 52% ethanol (*v*/*v*) proved suitable in all experiments. The three purest 10 mL fractions combined yielded 12.21 mg spilanthol at 71.65% purity from NADES extract ChCl/P (choline chloride/1,2-propanediol, molar ratio 1:2, +20% m/m water). Ethanolic extract samples showed purities ranging from 77.27 to 80.27% in combined raw fractions. For all samples, purity increased by removing non-soluble substances from organic solutions. Pooled NADES extract fractions showed 89.71% in final samples, ethanolic extracts 87.25 to 91.93%. The highest purities of individual fractions per extract were 89.23 to 94.15%. This cheap and simple purification process is promising to acquire spilanthol for research purposes or as a sample preparation step before HPLC on a semi-preparative to preparative scale, as the substance is highly priced and scarcely available on the market. Organic solvents can be reused, and preliminary scale-up possibilities are shown.

Keywords: *Acmella oleracea*; spilanthol; NADES; ethanol; green extraction; HPLC-DAD; isolation; SPE; NMR

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1. Introduction

Spilanthol (2E,6Z,8E-*N*-isobutyl-2,6,8-decatrienamide, Figure 1), a natural compound belonging to the group of *N*-alkylamides, is a major constituent of *Acmella oleracea* (L.) R.K. Jansen [1–4]. Alkylamide-rich extracts and purified spilanthol are of pharmacological interest for antinociceptive and analgesic effects [5–9], diverse antimicrobial [10,11], antimalarial [12], antifungal [13], and anti-inflammatory activity [14,15], but also investigated for cosmetic applications [16]. Persistent research into these therapeutic effects emphasizes the importance of successful spilanthol extraction and isolation.

Emerging from traditional extraction methods like Soxhlet extraction and maceration [17–19], there are previously described modern extraction methods for spilanthol, like supercritical carbon dioxide extraction [20–22] and microwave or ultrasound-assisted solvent extractions [23]. Extending this field of research, we recently showed the applicability of NADES (natural deep eutectic solvents) as extraction agents for spilanthol from *Acmella*

oleracea [24]. NADES are eutectic mixtures of naturally occurring hydrogen bond acceptors and donors. Physiologically, they could be major facilitators in the biosynthesis and storage of natural products in living cells, posing as an additional solvent phase to water and lipid [25]. The potential of NADES as interesting green extraction media for natural products has been widely recognized, reflected in numerous publications in recent years [26–28]. However, the purification of target compounds from NADES poses a challenging step after successful extraction, as their components are non-volatile. Several different techniques have found application, like solid–liquid extractions on adsorption resins, liquid–liquid extractions using aprotic solvents, or the addition of antisolvents to precipitate the desired target compound [29].

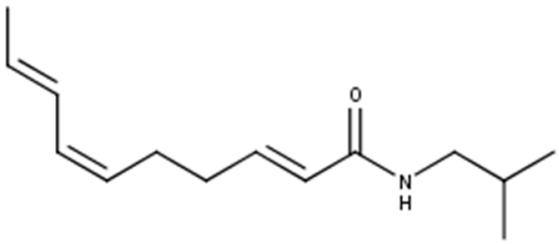

Figure 1. Structure of spilanthol (2E,6Z,8E-*N*-isobutyl-2,6,8-decatrienamide), the main alkylamide component in *Acmella oleracea* (L.) R.K. Jansen.

The objective of this study is to investigate a different approach for spilanthol isolation from NADES and ethanolic *Acmella oleracea* flower head extracts in a simple and green method with minimal instrumental effort. For this, extracts are subjected to SPE (solid-phase extraction), eluting merely with ethanolic solutions. This proposed alternative to aforementioned purification techniques builds on a simple reversed-phase chromatographic process, using commercially available SPE columns. It is therefore easy to use and shows potential for modification according to different applications in natural product retrieval from NADES. The choice of NADES investigated is based on our previous findings, where combinations of choline chloride with 1,2-propanediol, methylurea, or citric acid showed promising extraction results [24]. Determination of the relative distribution of spilanthol in SPE fractions is performed by HPLC-DAD. Quantification and investigation of purity follows in an NMR-based approach. The successful purification of spilanthol in a fast and inexpensive way without complex instrumentation would expand possibilities for further research, as this substance is highly priced and to-date scarcely available on the market.

2. Materials and Methods

2.1. Plant Material

Acmella oleracea (L.) R.K. Jansen plants were raised from seeds (Rühlemann, Horstedt, Germany) and then cultivated in a greenhouse pot culture at the Botanical Garden, University of Graz, from May to September of 2019. Flower heads were continually harvested, dried at room temperature, and stored in brown glass containers until further investigation in extraction experiments. The plant material was ground in a Retsch ZM100 centrifugal mill with 0.5 mm mesh (Retsch, Haan, Germany) before extractions. Voucher specimens are deposited at the Department of Pharmacognosy, University of Graz.

2.2. Chemicals and Solvents

Chemicals for NADES preparations were choline chloride (98%, Thermo Fisher Scientific, Waltham, MA, USA), 1,2-propanediol (98%, Thermo Fisher Scientific, Waltham, MA, USA), methylurea (97%, Acros Organics, Geel, Belgium) and citric acid monohydrate (99.5%, Carl Roth, Karlsruhe, Germany). Further solvents used were ethanol (96% *v*/*v*, partially denatured with 1% methyl ethyl ketone, AustrAlco, Spillern, Austria) and dichloromethane (≥99.5%, Carl Roth). For HPLC mobile phases, ultrapure water was

prepared with a Barnstead MicroPure system (Thermo Fisher Scientific, Waltham, MA, USA) and used in combination with HPLC-grade acetonitrile (VWR, Radnor, PA, USA) and formic acid (Honeywell, Charlotte, NC, USA). NMR analyses were performed in solvent chloroform-d_1 (99.80% deuteration grade, VWR, Leuven, Belgium). Reference substance spilanthol was isolated as described in our previously published work [24]. All percentage values for ethanol in water are given as volume per volume (% *v*/*v*).

2.3. Preparation and Synthesis of NADES

Three NADES combinations were prepared with choline chloride as a hydrogen bond acceptor (HBA) and 1,2-propanediol (ChCl/P), citric acid monohydrate (ChCl/C), or methylurea (ChCl/MeU) as hydrogen bond donor (HBD) in a molar ratio of 1:2 with water addition of +20% m/m for a reduction in viscosity. NADES components were weighed into Erlenmeyer flasks taking into account the desired molar ratio. For NADES preparations, mass ratios were calculated from molar ratios of choline chloride (139.626 g/mol), citric acid monohydrate (210.138 g/mol), methylurea (74.083 g/mol), and 1,2-propanediol (76.095 g/mol). For extracts in semiquantitative purification experiments, batches of 40 g of combined NADES components with 8 g of water addition were prepared. The synthesis of solvent ChCl/P was scaled up for the extraction of spilanthol in isolation experiments. Preparation of 150 mL of ChCl/P was necessary; therefore, the volume of the first batch of ChCl/P was determined (43.9 mL) and masses of individual components scaled up accordingly by a factor of 3.417. Syntheses of homogenous, clear solvents were performed according to a heating–stirring approach in sealed Erlenmeyer flasks in a water bath at 50 °C.

2.4. Extraction Methods

2.4.1. Extractions with NADES

For semi-quantitative purification experiments, NADES extracts were prepared from 1 g of plant material per 10 mL of solvent in plastic tubes. Mixtures were dispersed manually with a spatula for 5 min and sealed before extraction in a Memmert Ulm 500 incubator (Memmert, Schwabach, Germany) for 60 min at 50 °C. After extraction, 30 mL of water were added (1:4 dilution) to further decrease viscosity and facilitate the separation of a clear supernatant from plant material by using a small plug of cotton wool and compressing the mixture manually. A volume of 20 mL of this dilution was used for purification of spilanthol with solid-phase extraction (SPE). The dilution was checked for volume contraction, which was found to be non-existent. Therefore, 20 mL of dilution in SPE were equivalent to the separation of 5 mL of raw NADES extract, prepared from 0.5 g of plant material.

For isolation of spilanthol with SPE, the NADES extraction was scaled up to 15 g of plant material in 150 mL of solvent. It was performed in a sealed Erlenmeyer flask under constant stirring on a heating plate at 50 °C for 60 min. The extract was diluted with 450 mL of water (1:4) and filtered through a paper filter. A volume of 400 mL were used for SPE and consecutively applied to the column in eight portions of 50 mL. In total, this was equivalent to the separation of 100 mL of raw NADES extract prepared from 10 g of plant material. For each reported SPE separation, one individual NADES extract sample was prepared.

2.4.2. Extraction with Ethanol

An amount of 20 g of plant material was macerated at room temperature with 400 mL of 96% ethanol in a sealed Erlenmeyer flask for 24 h. Extraction was performed under light exclusion and enhanced by constant stirring. The raw extract was filtered through a paper filter and evaporated to dryness using a rotary evaporator at a temperature of 40 °C. The resinous dry extract had a mass of 3.69 g, equaling a drug extract ratio of 5.42 to 1. Individually sampled portions of this dry extract were used for SPE separation experiments.

2.5. Solid-Phase Extraction (SPE) Methods

All SPE experiments were performed on Chromabond C18 columns without endcapping (Macherey-Nagel, Düren, Germany) with a sorbent mass of 10 g and a column volume of 70 mL. Processing under vacuum was achieved with a Biotage VacMaster manifold (Biotage, Uppsala, Sweden).

2.5.1. SPE for the Recovery of Spilanthol from NADES Extracts

For the purification of spilanthol from NADES extracts, SPE columns were activated with 150 mL of ethanol and equilibrated with 150 mL of water. NADES extract dilutions were then loaded in volumes of 20 mL for semi-quantitative experiments and 400 mL for isolation. NADES components were washed out with 50 mL of water, before fractionation of extracts with 150 mL of 52% ethanol in 10 mL steps. Fractions were collected in 10 mL volumetric flasks. Finally, the column was washed with 50 mL of 96% ethanol. For preceding method development, additional ethanol concentrations were used in the fractionation step. They ranged from 10 to 90% ethanol with 10% increments and 50 to 60% ethanol with 2% increments in respective experiments.

2.5.2. SPE for the Recovery of Spilanthol from Ethanolic Extracts

The SPE purification of spilanthol from ethanolic extracts differed from NADES experiments in the equilibration step, which was performed with 150 mL of 52% ethanol. This was necessary due to the isocratic fractionation with 52% ethanol, and the resulting need for solution of dry ethanolic extract samples in the same starting conditions. Dry ethanolic extract was dissolved in 52% ethanol in different concentrations and centrifuged for 5 min at 4000 rpm (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). For semi-quantitative experiments, an equivalent of 100 mg of dry extract was loaded onto the column (10 mL, 10 mg/mL). For isolation, 500 mg (20 mL, 25 mg/mL) and 1 g (30 mL, 33.3 mg/mL) were applied. No wash step was performed after loading the column, because solvents in sample preparation and fractionation were identical. Therefore, in contrast to NADES extracts, no flushing of solvent components was necessary prior to elution. Isocratic fractionation and final washing of the column was identical to experiments with NADES extracts.

2.6. HPLC-DAD Analysis for Relative Quantification of Spilanthol in SPE Fractions

The HPLC in use for the analysis of SPE fractions was a Vanquish Core System (Thermo Fisher Scientific) with VC-P20-A quaternary pump, VC-A12-A autosampler, VC-C10-A column compartment and VC-D11-A diode array detector. Chromatography was performed on a Zorbax Eclipse Plus C18 column, 2.1 × 100 mm and 1.8 µm particle size (Agilent, Santa Clara, CA, USA) as a stationary phase, with a mobile phase of water + 0.1% formic acid (A) and acetonitrile (B). Gradient elution was performed with initial 10% B, a linear increase to 90% B at 20 min and up to 100% B at 20.5 min. At 23 min a return to starting conditions was initialized and 10% B maintained from 23.5 to 33 min for re-equilibration before the next injection. Further parameters were as follows: a flow rate of 0.25 mL/min; a column temperature of 35 °C. DAD-UV detection was performed from 190 to 500 nm, with an extracted wavelength for spilanthol quantification at 230 nm, and 210 nm for purity determination in fractions. A calibration curve for spilanthol in a linear working range of 10–500 µg/mL was used as presented in our preceding work [24].

Before sampling for HPLC, SPE fractions were adjusted to the same exact volume. Injection volumes varied between 0.5–10 µL for individual samples to reach desired signal intensity, i.e., peak areas for spilanthol within the calibrated linear working range. Signals were then multiplied to represent the full volume of each fraction and normalized, with added peak areas of all fractions equaling 100%. This approach enabled direct comparison of spilanthol distribution between different SPE experiments. All signals for spilanthol in separate SPE fractions were used for relative quantification and distribution analysis of the target compound. The purest fractions were identified in two levels, where peak areas for

spilanthol in UV chromatograms at 210 nm accounted for either >95% or >98% of total in a retention time range of 3–25 min. Additionally, 3D-chromatograms in a wavelength range of 190–500 nm were evaluated. More detailed information was obtained by NMR analysis after scale-up experiments.

2.7. NMR Analysis for Purity Determination and Quantification of Isolated Spilanthol

NMR spectra were recorded using an Avance NEO 400 MHz spectrometer (Bruker, Rheinstetten, Germany), the experimental temperature was 25 °C, and the solvent was $CDCl_3$. A data set consisting of ^{1}H, ^{13}C, COSY, HSQC, and HMBC experiments was recorded for spilanthol. For each of the twelve extract samples in scale-up experiments, a proton spectrum was recorded with a relaxation delay of 10 s and an acquisition time of 1.25 s. Compounds were dissolved in 720 µL $CDCl_3$.

2.8. Statistical Analysis

Mean values and standard deviations were calculated in Microsoft Excel version 2407.

3. Results and Discussion

3.1. General Method Development for the SPE Elution of Spilanthol with Ethanol

Several preliminary tests using NADES extracts helped to define the elution characteristics of spilanthol in the method development stage for SPE purification. In these tests, step elutions with 10% increments in ethanol concentration from 10 to 90% showed spilanthol to elute only in the fraction produced with 60% ethanol. An additional experiment with 2% increments in elution from 50% to 60% showed spilanthol in fractions with 52% and 54% ethanol. These solvent concentrations were further investigated and compared in isocratic experiments. Figure 2 depicts the relative amount of spilanthol eluted from an extract produced with NADES ChCl/MeU in 15 fractions, each produced with 10 mL of solvent 52% ethanol or 54% ethanol. Fraction 16 represents the wash step with 50 mL of 96% ethanol, to show residual spilanthol and clean the column of more lipophilic accompanying substances if re-use of the same column is desired.

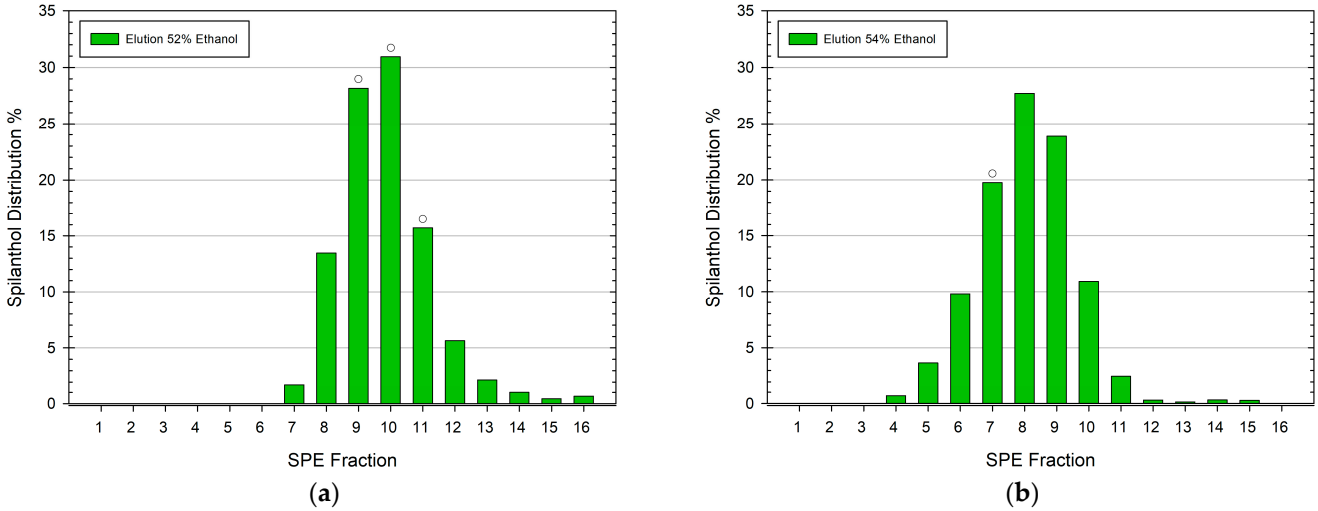

Figure 2. Relative distribution of spilanthol in SPE fractions of 5 mL NADES extract ChCl/MeU eluted with (**a**) 52% ethanol (fractions 1–15) or (**b**) 54% ethanol (fractions 1–15) and a 96% ethanol wash step (fraction 16). ○ Peak area > 95% in HPLC-UV at 210 nm.

As expected, spilanthol eluted faster with 54% ethanol and could be detected from fraction 4 onwards, while it was first present in fraction 7 with 52% ethanol. However, more focused elution could be observed with 52% ethanol, where 28.2 and 30.9% of spilanthol appeared in fractions 9 and 10, respectively. To illustrate this, Figure 3 shows the chro-

matogram used for spilanthol quantification at 230 nm for the elution with 52% ethanol, fraction 10.

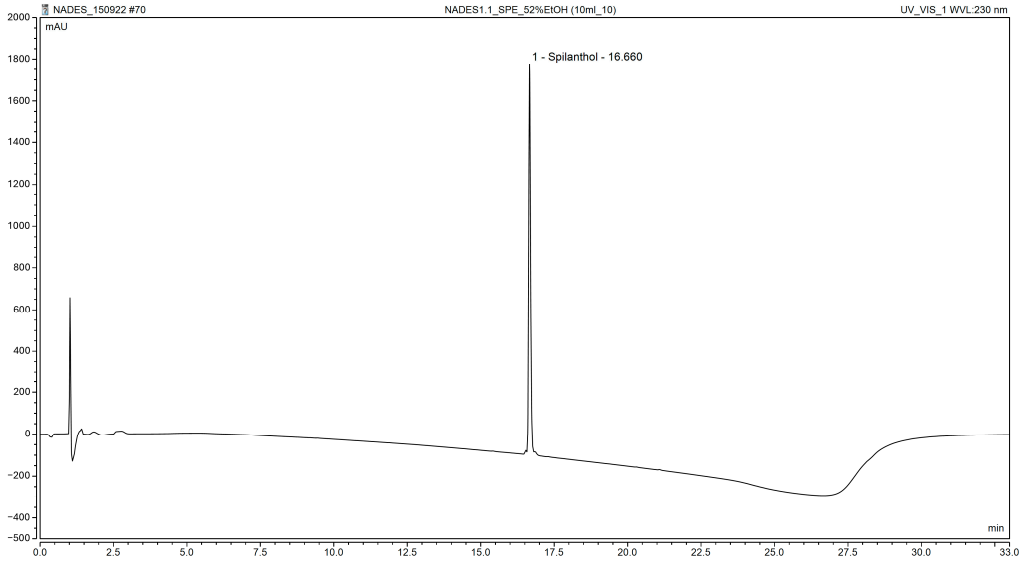

Figure 3. HPLC-UV chromatogram (230 nm) of extract ChCl/MeU SPE fraction 10, eluted with 52% ethanol.

The chromatogram given in Figure 3 depicts the highest intensity of spilanthol for the SPE elution of extract ChCl/MeU with 52% ethanol and can be seen as representative for all further spilanthol quantifications in SPE fractions. When eluting with 54% ethanol, 27.7 and 23.9% of spilanthol were present in fractions 8 and 9. Only fraction 7 of 54% ethanol could be classified as pure according to HPLC-DAD, whereas fractions 9 to 11 of 52% ethanol showed high purity. An accompanying substance with a retention time of 17.5 min was present in all ChCl/MeU fractions that were high in spilanthol. 3D chromatograms are given in Supplementary Materials (Figures S1 and S2). A concentration of 52% ethanol was determined as isocratic eluent for subsequent semi-quantitative and isolation experiments.

3.2. Spilanthol Distribution in SPE Fractions of Different NADES Extracts

The isocratic fractionation with 52% ethanol was separately applied to different NADES extracts as a proof of concept and to determine possible influences of the NADES solvent on SPE separation. Figure 4 shows comparative results for the fractionation of three different NADES extracts (5 mL diluted with 15 mL of water), produced with ChCl/MeU, ChCl/C and ChCl/P. The relative distribution of spilanthol in 15 fractions of 52% ethanol (10 mL) and a final wash step with 96% ethanol (50 mL) is given.

52% ethanol was confirmed as suitable eluent for the fractionation of different NADES extracts. Traces of spilanthol were first detected in fraction 6 of ChCl/C and ChCl/P, as well as fraction 7 of ChCl/MeU. Similar elution characteristics were observed for ChCl/C and ChCl/P with the purest fractions 9, 10 and 11 accounting for 78.3% and 75.1%, respectively. Overall, spilanthol eluted in earlier fractions of ChCl/MeU, but again the purest in fractions 9, 10 and 11, representing 74.8% in relative distribution. ChCl/MeU consistently showed traces of adulteration with a retention time of 17.5 min in HPLC of the purest fractions, that eluted close to spilanthol (16.6 min). ChCl/C proved most difficult to handle due to higher viscosity. These observations led to the decision, that ChCl/P was most suited for scale-up experiments. 3D chromatograms of NADES fractions can be found in Supplementary Materials (Figures S1, S3 and S4).

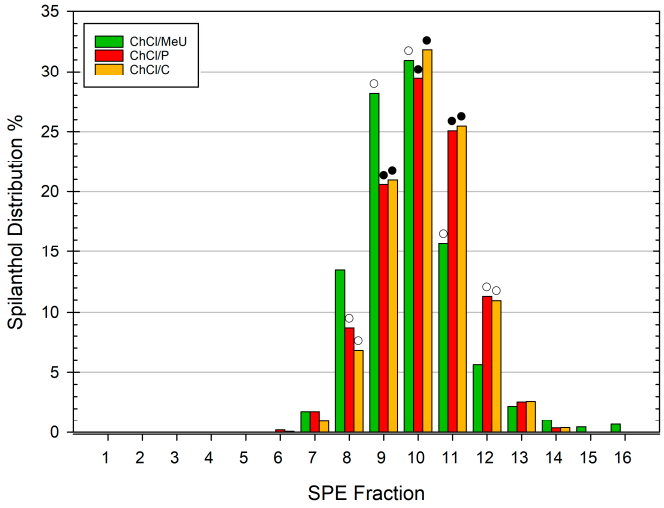

Figure 4. Relative distribution of spilanthol in SPE fractions of 5 mL NADES extracts ChCl/MeU, ChCl/P and ChCl/C eluted with 52% ethanol (fractions 1–15) and a 96% ethanol wash step (fraction 16). ○ Peak area > 95% in HPLC-UV at 210 nm. • Peak area > 98% in HPLC-UV at 210 nm.

3.3. Spilanthol Distribution in SPE Fractions of Ethanolic Extracts

In an additional approach towards simple spilanthol isolation, SPE purification through isocratic fractionation was also tested on dry ethanolic extracts. Only ethanol and water were needed as solvents throughout this procedure. An amount of 100 mg of ethanolic extract were applied to the SPE-column as solution in 52% ethanol (EtOH 100 mg, 10 mg/mL) and separated into 15 fractions (10 mL) using the same solvent, plus a final wash step with 96% ethanol (50 mL). Results are given in Figure 5.

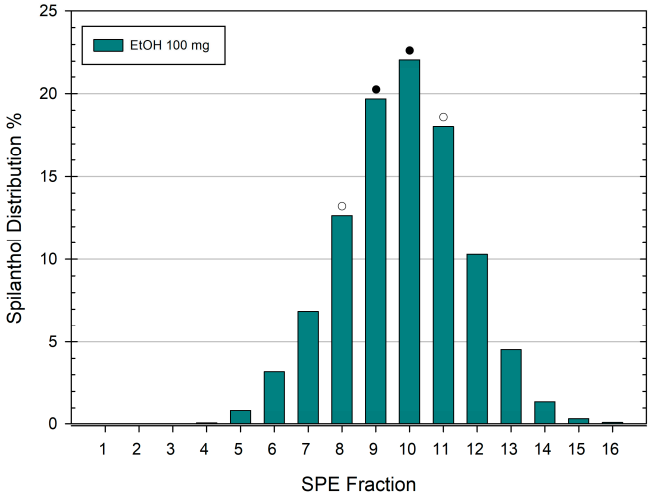

Figure 5. Relative distribution of spilanthol in SPE fractions of 100 mg ethanolic extract eluted with 52% ethanol (fractions 1–15) and a 96% ethanol wash step (fraction 16). ○ Peak area > 95% in HPLC-UV at 210 nm. • Peak area > 98% in HPLC-UV at 210 nm.

Spilanthol first eluted in fraction 4. This faster elution compared to NADES extracts was expected since the ethanol concentration within the sample solution already facilitated mobility of the target on column in the loading step. Distribution was also broadened over more fractions, with spilanthol detected from fractions 4 to 16. Fractions 9 and 10 showed highest purity in HPLC-DAD, which accounted for 41.8% of spilanthol in relative distribution (Supplementary Materials, Figure S5). This experiment showed that it is possible to purify spilanthol with only ethanol needed as organic solvent from extraction to fractionation.

3.4. Scale-Up and Isolation of Spilanthol from NADES and Ethanolic Extracts

Since SPE purification was successful on a semi-quantitative scale for NADES and ethanolic extracts, investigation of scale-up possibilities and isolation of the target compound was the next logical step. The scale-up for the separation of NADES extracts was performed by loading the SPE column with 100 mL of an extract produced with ChCl/P diluted with 300 mL of water. For dry ethanolic extracts, 500 mg (EtOH 500 mg, 25 mg/mL) and 1000 mg (EtOH 1000 mg, 33.3 mg/mL) were applied in 52% ethanol solution. The separation of 1000 mg dry extract was also repeated to acquire additional data (EtOH 1000 mg (1) and (2)). SPE fractionation was performed in analogy to previous experiments (3.3). Figure 6 shows the relative distribution of spilanthol in fractions produced in scale-up experiments.

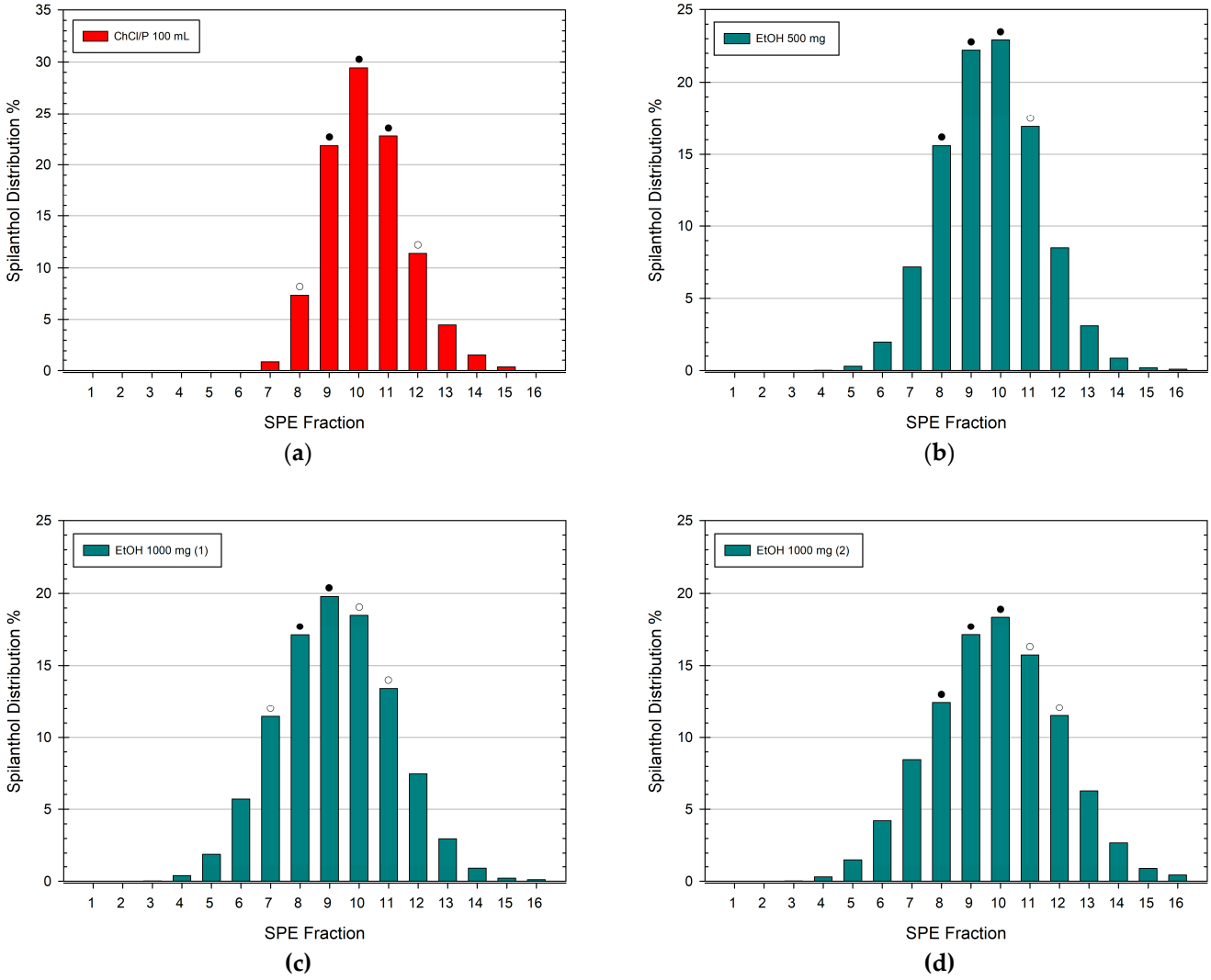

Figure 6. Relative distribution of spilanthol in SPE fractions of extracts in isolation experiments eluted with 52% ethanol (fractions 1–15) and a 96% ethanol wash step (fraction 16). (**a**) A volume of 100 mL NADES extract ChCl/P; (**b**) 500 mg ethanolic extract; (**c**,**d**) 1000 mg ethanolic extract. ○ Peak area > 95% in HPLC-UV at 210 nm. • Peak area > 98% in HPLC-UV at 210 nm.

Elution characteristics did not differ between semi-quantitative and quantitative experiments with NADES ChCl/P (Figures 4 and 6a). The purest fractions in HPLC-DAD were 9, 10 and 11, resulting in 74.1% of spilanthol in relative distribution across fractions (Supplementary Materials, Figure S6). Highest concentration of spilanthol was observed in fraction 10 at 29.4% of total.

Purification of spilanthol was also successful with all higher loads of dry ethanolic extract. The relative distribution of spilanthol in fractions was largely consistent between semi-quantitative and quantitative experiments (Figures 5 and 6b–d). A slight shift towards lower fractions only occurred with sample EtOH 1000 mg (1) (Figure 6c), where the purest fractions 8 and 9 accounted for 36.9% of spilanthol. Other scale-ups showed the purest fractions in HPLC-DAD to be 8, 9 and 10, suggesting satisfactory robustness of the SPE separation (Figures S7–S9). Combined peak areas for spilanthol in these fractions equaled 60.7% for EtOH 500 mg and 48.0% for EtOH 1000 mg (2). Higher loads of ethanolic extract led to broadening of spilanthol distribution across fractions.

For better comparison of extract separation with different loads, results for combined SPE fractions with standard deviation for samples of ethanolic extracts and NADES ChCl/P extracts are given in Figure 7a,b, respectively.

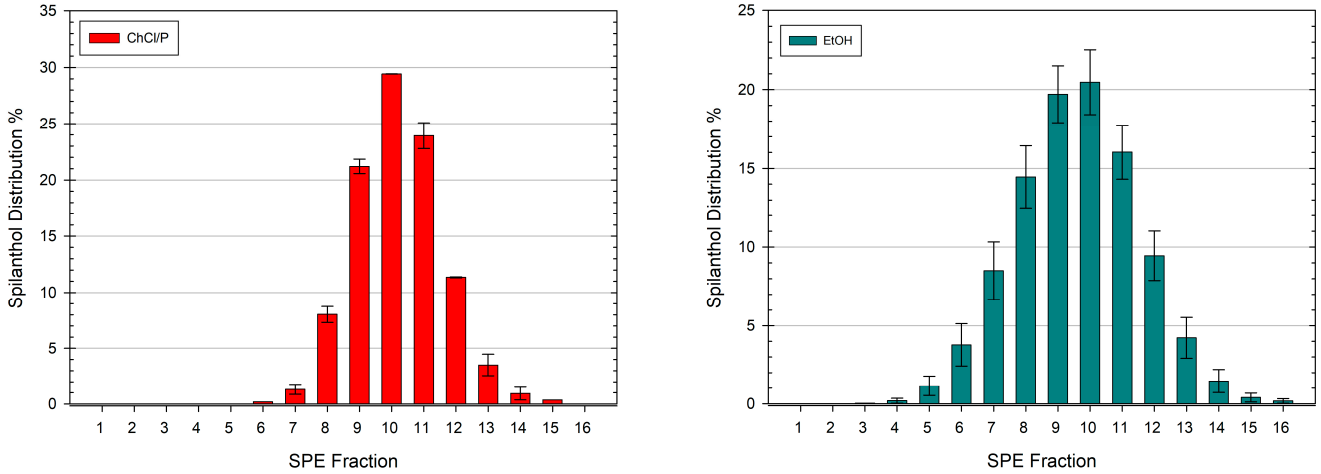

Figure 7. Relative distribution of spilanthol in SPE fractions of two ChCL/P extracts (5 mL, 100 mL) (**a**) and four ethanolic extract samples (100 mg, 500 mg, and 1000 mg (sample 1 and sample 2)) (**b**).

For most relevant fractions 9 to 11 of ChCl/P, spilanthol distribution varied by a standard deviation of 0.6% (fraction 9), 0.01% (fraction 10) and 1.1% (fraction 11) in results for the separation of 5 mL and 100 mL of NADES extract (Figure 7a). ChCl/P separation showed remarkable robustness when loading higher extract volumes onto the SPE column. For ethanolic extract fractions 8 to 10, differences were more pronounced, mostly due to the broadening of spilanthol distribution with higher ethanolic extract loads and the described shift towards lower fractions with sample EtOH 1000 mg (2). Here, spilanthol distribution varied by a standard deviation of 2.0% (fraction 8), 1.8% (fraction 9) and 2.1% (fraction 10), evaluating combined results for 100 mg, 500 mg, 1000 mg (1) and 1000 mg (2) (Figure 7b).

3.5. Purity and Quantification of Isolated Spilanthol

To transfer from relative distribution values to absolute quantification, an NMR-based approach was used for determination of the quantity and purity of isolated spilanthol. A similar method has been previously postulated by Grymel et al. [19], comparing the integration values of characteristic spilanthol proton signals with those of a standard. Our method involved the comparison with the proton signal of $CHCl_3$ in the deuterated chloroform. Twelve spilanthol-rich fractions were investigated, representing the three purest fractions of each scale-up experiment. Figure 8 shows an exemplary proton spectrum for spilanthol quantification in sample EtOH 500 mg, fraction 9.

The integral of the chloroform proton signal in this sample represents 18 µmol protonated solvent, as the known protonation grade of the solvent is 0.2%. The integral of the methylene group C-1′ (two protons) was used to determine the molar amount (14 µmol) of spilanthol in this sample. NMR data for spilanthol are given in Supplementary Materials (Table S1).

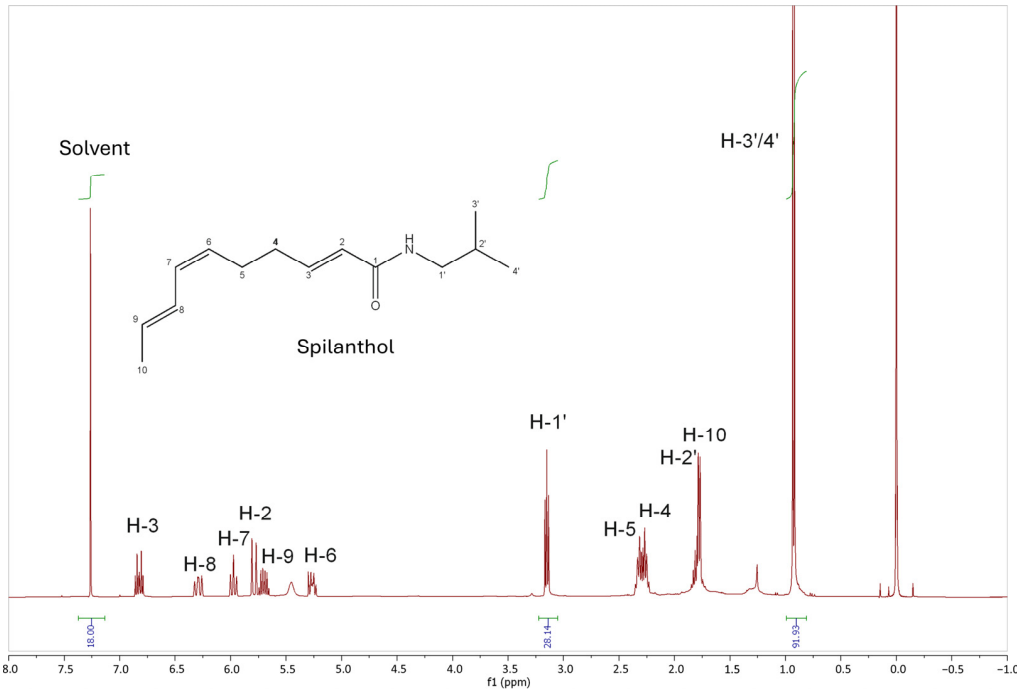

Figure 8. Proton spectrum (400 MHz) of sample EtOH 500 mg, Fraction 9 in 720 µL of solvent $CDCl_3$.

For sample preparation, target fractions were evaporated to dryness on a rotary evaporator, completely dispersed in dichloromethane with the help of an ultrasonic bath and transferred to glass vials. In this first solution step, particles were visible in the transferred sample solutions, which were obviously not soluble in the pure organic solvent. Samples were evaporated under nitrogen and weighed without attempts of further purification to document the total mass of dry SPE fractions. However, since these impurities could not consist of the target alkylamide spilanthol due to its high solubility in dichloromethane, organic solution and removal of the precipitate could be used as a further purification step after SPE. Confirmation was attempted during NMR analysis, as dissolving in deuterated chloroform also left the same insoluble impurities. After transfer of solutions without precipitate to NMR tubes and measurements, clear samples were evaporated and weighed again. The mass of spilanthol determined by NMR in each sample could therefore be related to initial sample masses including insoluble adulterants and final sample masses representing only organic substances that were captured by NMR spectroscopy, resulting in two sets of percent values for spilanthol purity. Table 1 lists quantified spilanthol in each sample, initial and final sample masses, and calculated spilanthol purity for both cases.

Extract ChCl/P yielded a total of 12.21 mg of spilanthol across three raw SPE fractions. NMR analysis revealed the purity in combined initial samples to be 71.65%. This seemed to stand in contrast to the evaluation of purity in HPLC chromatograms, where no significant adulterants were detected. It is possible, that this is due to impurities, which showed no UV absorbance or no retention in reversed-phase HPLC and therefore remained undetected. It is also emphasized that mere HPLC control of purity can suggest misleading results without NMR confirmation. Another contributing factor was suspected in the integrity of SPE column material during elutions. To test this assumption, a column was eluted under identical conditions as used in separation experiments, however without loading any extract sample. After drying, a white residue remained, that could only come from stationary-phase material in SPE, or inorganic impurities within the solvents used for elution. This would coincide with insoluble residues observed during experiments.

The separation of ethanolic extracts showed a higher spilanthol purity of combined initial samples, ranging from 77.27 to 80.27%. Spilanthol yields fluctuated, with 8.62 mg purified from EtOH 500 mg, as well as 15.46 mg and 10.30 mg from EtOH 1000 mg (1) and (2), respectively. Lower yields of spilanthol in EtOH 1000 mg (2) could be explained by

variances in sampling of dry extract. It could be expected that the resinous raw extract is not completely homogenous after evaporation of the extraction agent ethanol, therefore leading to not directly comparable yields between individually sampled portions. Further investigations must follow up on these findings in the future.

Table 1. Spilanthol quantity in SPE fractions as determined by NMR, sample weights and purity.

Extract Source, SPE	Spilanthol [1] [mg]	Initial Sample [2] [mg]	Spilanthol Purity [%]	Final Sample [3] [mg]	Spilanthol Purity [%]
ChCl/P, Fraction 9	3.62	6.44	56.21	4.27	84.78
ChCl/P, Fraction 10	4.73	5.99	78.96	5.24	90.27
ChCl/P, Fraction 11	3.86	4.61	83.73	4.10	94.15
Total	12.21	17.04	71.65	13.61	89.71
500 mg Ethanolic Extract, Fraction 8	2.40	3.21	74.77	2.94	81.63
500 mg Ethanolic Extract, Fraction 9	3.11	3.78	82.28	3.52	88.35
500 mg Ethanolic Extract, Fraction 10	3.11	3.94	78.93	3.42	90.94
Total	8.62	10.93	78.87	9.88	87.25
1000 mg Ethanolic Extract (1), Fraction 8	4.97	6.37	78.02	5.73	86.74
1000 mg Ethanolic Extract (1), Fraction 9	5.52	6.59	83.76	6.29	87.76
1000 mg Ethanolic Extract (1), Fraction 10	4.97	6.30	78.89	5.57	89.23
Total	15.46	19.26	80.27	17.59	87.89
1000 mg Ethanolic Extract (2), Fraction 8	3.11	4.07	76.41	3.57	87.11
1000 mg Ethanolic Extract (2), Fraction 9	3.37	4.64	72.63	3.62	93.09
1000 mg Ethanolic Extract (2), Fraction 10	3.82	4.62	82.68	4.08	93.63
Total	10.30	13.33	77.27	11.27	91.39

[1] Quantity determined by NMR; [2] dry weight of raw SPE fractions; [3] dry weight of clear organic solution after NMR.

Each extract sample showed one fraction of spilanthol purity above 82% in initial samples. For all samples, purity increased after separating an organic solution from non-soluble precipitation during the step of NMR measurements. The calculated purity of spilanthol in clear organic solutions after NMR was above 80% for all individual final samples. The highest purities of individual fractions per extract ranged from 89.23 to 94.15%. For ChCl/P, overall purity in final samples increased to 89.71%. The purification effect was most pronounced for fraction 9 of extract ChCl/P, with an increase in purity from 56.21 to 84.78%. Ethanolic extract samples gave purities for pooled final samples from 87.25 to 91.39% per extract. The most remarkable increase was shown for fraction 9 of 1000 mg (2) with 72.63 to 93.09%.

The purification of spilanthol from extracts using SPE, eluting with ethanolic solutions, held up well compared to separation techniques in the literature. Semi-preparative HPLC, though efficient, means high instrumental effort and is also dependent on the use of organic solvents like methanol or acetonitrile in high volumes [17,24]. Flash and open-column chromatography methods for the purification of spilanthol often use mixtures of hexane and ethyl acetate as the mobile phase, which can also not be classified as predominantly green chemistry approaches in natural product isolation [18,19,23]. Our method only involves an organic solvent other than ethanol in a final purification step.

Purity determination of final spilanthol samples reported in literature must also be met with caution, as results of HPLC-DAD-based methods are highly dependent on the evaluated wavelength in chromatograms. Reported purities range from >94% to 97% [18,21], and only seldom acknowledge these variations, e.g., by describing a purity of 100% at 270 nm and 96.6% at 210 nm [23]. NMR-based approaches for purity determination, as used in our study, are more reliable, with 97% spilanthol in samples reported previously [19]. In that study, a dry secondary extract containing 10% spilanthol served as starting material for purification by open column chromatography. This shows that source extracts must also be

considered, when discussing the achievable purity of spilanthol. For example, supercritical CO_2 extraction is highly effective, however, at extensive instrumental effort [21].

In light of these comparisons, our NADES and ethanolic extraction methods and purest SPE fractions, ranging from 89.23 to 94.15% spilanthol determined by NMR, offer an accessible, accurate and low instrumentation tool for alternative natural product extraction and purification.

4. Conclusions

The possibility to purify spilanthol from *Acmella oleracea* NADES and ethanolic extracts in a green process with minimal instrumental effort by SPE on C18 column material with 52% ethanol was shown for the first time. The three purest fractions could be determined by HPLC-DAD for each extract sample. NMR quantification of spilanthol after scale-up revealed a yield of 12.21 mg spilanthol from 150 mL of NADES extract ChCl/P at a purity of 71.65%. Spilanthol yields from ethanolic dry extract were 8.62 mg purified from 500 mg of extract (purity 78.87%), as well as 15.46 mg (purity 80.27%) and 10.30 mg (purity 77.27%) from two 1000 mg extract samples. These purifications only needed ethanol and water as solvents throughout the whole procedure from extract preparation to spilanthol sample.

During NMR analysis, adulterants insoluble in dichloromethane and deuterated chloroform were observed. Evaporation of clear organic solutions after NMR led to increased purities of 89.71% for ChCl/P and 87.25 to 91.39% for ethanolic extract samples. An additional organic solution step after SPE could be used to profoundly increase purity. Solvents in this step can be completely recovered and alternative solvents need to be further investigated.

Overall, the cheap, simple, and mostly green purification process could prove interesting for research purposes to acquire spilanthol in sufficient quantities for further testing, as this alkylamide is an interesting pharmacological target compound, yet to date only commercially available from a limited number of sources at a high price. SPE with 52% ethanol could also be applied as a fast and green sample preparation step before semi-preparative or preparative HPLC. Ethanol used throughout the procedure can easily be recovered and reused. For SPE, scale-up possibilities were explored with limitations of cartridge size at hand, but further investigations need to be undertaken to explore industrial scaling.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11080251/s1, Figure S1. 3D chromatograms (190–300 nm) of extract ChCl/MeU purest SPE fractions, eluted with 52% ethanol. (a) Fraction 9; (b) Fraction 10; (c) Fraction 11; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S2. 3D chromatogram (190–300 nm) of extract ChCl/MeU purest SPE fraction 7, eluted with 54% ethanol. M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S3. 3D chromatograms (190–300 nm) of extract ChCl/P purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; (e) Fraction 12; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S4. 3D chromatograms (190–300 nm) of extract ChCl/C purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; (e) Fraction 12; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S5. 3D chromatograms (190–300 nm) of extract EtOH 100 mg purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S6. 3D chromatograms (190–300 nm) of extract ChCl/P (100 mL) purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; (e) Fraction 12; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S7. 3D chromatograms (190–300 nm) of extract EtOH 500 mg purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S8. 3D chromatograms (190–300 nm) of extract EtOH 1000 mg (1) purest SPE fractions, eluted with 52% ethanol. (a) Fraction 7; (b) Fraction 8; (c) Fraction 9; (d) Fraction 10; (e) Fraction 11; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol. Figure S9. 3D chromatograms (190–300 nm) of extract EtOH 1000 mg

(2) purest SPE fractions, eluted with 52% ethanol. (a) Fraction 8; (b) Fraction 9; (c) Fraction 10; (d) Fraction 11; (e) Fraction 12; M: methyl ethyl ketone from partly denatured ethanol in samples; S: spilanthol; Table S1. Proton (100 MHz, $CDCl_3$) and carbon NMR data of spilanthol. The experimental temperature was 25 °C, and TMS was used as the internal standard.

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